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Effect Of N-3 Fatty Acids And Antioxidants On Oxidative Stress After Exercise

By: **STEVEN R. MCANULTY, DAVID C. NIEMAN**, MASHA FOX-RABINOVICH, VALERIE DURAN, LISA S. MCANULTY, DRU A. HENSON, FUXIA JIN, and MICHAEL J. LANDRAM

Abstract

Purpose: n-3 fatty acids are known to exert multiple beneficial effects including anti-inflammatory actions that may diminish oxidative stress. Supplementation with antioxidant vitamins has been proposed to counteract oxidative stress and improve antioxidant status. Therefore, this project investigated the effects of daily supplementation in 48 trained cyclists over 6 wk and during 3 d of continuous exercise on F2-isoprostanes (oxidative stress), plasma n-3 fatty acids, and antioxidant status (oxygen radical absorption capacity and ferric-reducing antioxidant potential). **Methods:** Cyclists were randomized into n-3 fatty acids (N3) (n = 11) (2000 mg of eicosapentaenoic acid and 400 mg of docosahexaenoic acid), a vitamin–mineral (VM) complex (n = 12) emphasizing vitamins C (2000 mg), E (800 IU), A (3000 IU), and selenium (200 Kg), a VM and n-3 fatty acid combination (VN3) (n = 13), or placebo (P) (n = 12). Blood was collected at baseline and preexercise and postexercise. A 4 x 3 repeated-measures ANOVA was performed to test main effects. **Results:** After exercise, F2-isoprostanes were higher in N3 (treatment effect $P = 0.014$). Eicosapentaenoic acid and docosahexaenoic acid plasma values were higher after supplementation (interaction effect $P = 0.001$ and 0.006 , respectively) in both n-3 supplemented groups. Oxygen radical absorption capacity declined similarly among all groups after exercise. Ferric-reducing antioxidant potential exhibited significant interaction ($P = 0.045$) and significantly increased after exercise in VN3 and VM ($P < 0.01$). **Conclusions:** This study indicates that supplementation with n-3 fatty acids alone significantly increases F2-isoprostanes after exhaustive exercise. Lastly, antioxidant supplementation augments plasma antioxidant status and modestly attenuates but does not prevent the significant n-3 fatty acid associated increase in F2-isoprostanes postexercise.

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Effect of n-3 Fatty Acids and Antioxidants on Oxidative Stress after Exercise

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¹Department of Health, Leisure, and Exercise Science, Appalachian State University, Boone, NC; ²Department of Family and Consumer Science, Appalachian State University, Boone, NC; ³Department of Biology, Appalachian State University, Boone, NC; and ⁴Chemistry, Appalachian State University, Boone, NC

ABSTRACT

McANULTY, S. R., D. C. NIEMAN, M. FOX-RABINOVICH, V. DURAN, L. S. McANULTY, D. A. HENSON, F. JIN, and M. J. LANDRAM. Effect of n-3 Fatty Acids and Antioxidants on Oxidative Stress after Exercise. *Med. Sci. Sports Exerc.*, Vol. 42, No. 9, pp. 1704–1711, 2010. **Purpose:** n-3 fatty acids are known to exert multiple beneficial effects including anti-inflammatory actions that may diminish oxidative stress. Supplementation with antioxidant vitamins has been proposed to counteract oxidative stress and improve antioxidant status. Therefore, this project investigated the effects of daily supplementation in 48 trained cyclists over 6 wk and during 3 d of continuous exercise on F₂-isoprostanes (oxidative stress), plasma n-3 fatty acids, and antioxidant status (oxygen radical absorption capacity and ferric-reducing antioxidant potential). **Methods:** Cyclists were randomized into n-3 fatty acids (N3) ($n = 11$) (2000 mg of eicosapentaenoic acid and 400 mg of docosahexaenoic acid), a vitamin–mineral (VM) complex ($n = 12$) emphasizing vitamins C (2000 mg), E (800 IU), A (3000 IU), and selenium (200 μ g), a VM and n-3 fatty acid combination (VN3) ($n = 13$), or placebo (P) ($n = 12$). Blood was collected at baseline and preexercise and postexercise. A 4×3 repeated-measures ANOVA was performed to test main effects. **Results:** After exercise, F₂-isoprostanes were higher in N3 (treatment effect $P = 0.014$). Eicosapentaenoic acid and docosahexaenoic acid plasma values were higher after supplementation (interaction effect $P = 0.001$ and 0.006 , respectively) in both n-3 supplemented groups. Oxygen radical absorption capacity declined similarly among all groups after exercise. Ferric-reducing antioxidant potential exhibited significant interaction ($P = 0.045$) and significantly increased after exercise in VN3 and VM ($P < 0.01$). **Conclusions:** This study indicates that supplementation with n-3 fatty acids alone significantly increases F₂-isoprostanes after exhaustive exercise. Lastly, antioxidant supplementation augments plasma antioxidant status and modestly attenuates but does not prevent the significant n-3 fatty acid associated increase in F₂-isoprostanes postexercise. **Key Words:** EICOSAPENTAENOIC ACID, DOCOSAHEXAENOIC ACID, F₂-ISOPROSTANES, CYCLING

Omega-3 fatty acids (n-3fa) comprise a family of unsaturated fatty acids that consist of α -linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), which are essential nutrients that must be obtained from food because of the inability of humans to synthesize these fatty acids *de novo* (28). Human beings evolved consuming a diet that contained about equal amounts of omega-6 (n-6fa) and n-3fa fatty acids. Current western diets provide ratios of n-6fa to n-3fa ranging from approximately 10:1 to 20:1 instead of the traditional range of 1:1 to 2:1, respectively. It is known that a high intake of n-6fa increases prothrombotic and proaggregatory activities

characterized by increases in blood viscosity and vasospasm and vasoconstriction (27). Given that n-3fa exhibit anti-inflammatory, antithrombotic, antiarrhythmic, hypolipidemic, and vasodilatory properties, an increased consumption of n-3fa would likely be beneficial (27).

Although n-3fa have potential benefits against certain diseases such as cardiovascular disease, Alzheimer's disease, Parkinson's disease, diabetes mellitus, essential hypertension, and atherosclerosis, questions remain about the effect on subsequent oxidative stress if supplemented alone or in conjunction with other antioxidants (4,8,15,26). Specifically, increased consumption of n-3fa may lead to increased lipid peroxidation (18). Thus far, the data obtained *in vivo* are not conclusive for (18) or against (1) increased lipid peroxidation with n-3 fatty acid supplementation. Furthermore, a study that has examined n-3fa supplementation and oxidative damage with exercise has not been conducted to our knowledge. Therefore, it is not known whether heavily exercising athletes should supplement with n-3fa.

It is well established that a causal link exists between oxidative stress and various disease states such as cancer and heart disease (2,7). Routine exercise is generally regarded as

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being beneficial and helpful in disease and mortality prevention (5). Somewhat paradoxical is the fact that acute exercise results in an increase in oxidative damage (17), but chronic exercise is known to be anti-inflammatory and reduces the amount of damage that occurs during acute exercise (12). Because an excess of oxidative stress is associated with negative consequences, supplementation with antioxidant vitamins has been proposed to counteract oxidative stress and to improve antioxidant status, but the existing literature is equivocal regarding exercise and antioxidant vitamins (16,17). Interestingly, Ristow et al. (25) found that supplementation with antioxidant vitamins actually ameliorated beneficial adaptations from exercise in preventing insulin resistance. Previous antioxidant research by our team indicates that individual antioxidants, even in high doses, are not effective at preventing peroxidation (17,23), but utilization of antioxidant combinations has shown more promise (16).

Because to our knowledge no study has yet been published that directly examines n-3 fatty acid supplementation and direct markers of oxidative damage after exhaustive exercise, the primary purpose of this project was to investigate whether supplementation would increase or decrease F_2 -isoprostanes. F_2 -isoprostanes are bioactive compounds that are considered to be the gold standard for determination of oxidative stress. Interestingly, F_2 -isoprostanes are elevated in obesity and various other disease states associated with states of elevated oxidative stress (20). Second, we wished to examine whether antioxidant vitamins might directly attenuate F_2 -isoprostanes when used as the only supplement or reduce potential increases in F_2 -isoprostanes associated with n-3fa peroxidation. We hypothesized that n-3fa and antioxidant vitamins would reduce oxidative stress and enhance antioxidant status.

METHODS

Subjects. Forty-eight trained cyclists were recruited and randomized into vitamin–mineral ($n = 12$) (VM), n-3fa ($n = 11$) (N3), vitamin and n-3fa combination ($n = 13$) (VN3), or placebo ($n = 12$) (P) groups. Written informed consent was obtained from each subject, and the experimental procedures were approved by the institutional review board of Appalachian State University.

Research design. Before the start of supplementation, subjects reported to the University Human Performance Laboratory for baseline orientation, blood sampling, and measurement of cardiorespiratory fitness. $\dot{V}O_{2\max}$ was determined using a graded maximal protocol (25 W increase every 2 min starting at 150 W), with the subjects using their own bicycles on CompuTrainer™ Pro Model 8001 trainers (RacerMate, Seattle, WA). Oxygen uptake and ventilation were measured using the MedGraphics CPX metabolic system (MedGraphics Corporation, St. Paul, MN). HR was measured using a chest HR monitor (Polar Electro Inc., Woodbury, NY). Basic demographic and training data

were obtained through a questionnaire. Body composition was assessed by hydrostatic weighing using an electronic load cell system (Exertech, Dresbach, MN), estimated residual volume, and the Siri equation.

Subjects agreed to avoid the use of large-dose VM supplements (above 100% of recommended dietary allowances), herbs, and medications known to affect oxidative stress during the entire study. During orientation, a dietitian instructed the subjects to follow a diet moderate in carbohydrate (using a food list) during the weekend before and 3 d of intensified exercise period. At 12:00–12:30 p.m. before each 3-h cycling bout, subjects ingested a standardized liquid meal (Boost Plus; Mead Johnson Nutritionals, Evansville, IN) at an energy level of $63 \text{ kJ}\cdot\text{kg}^{-1}$ ($15 \text{ kcal}\cdot\text{kg}^{-1}$). Boost Plus is a nutritionally complete, high-energy oral supplement with an energy density of $6.4 \text{ kJ}\cdot\text{mL}^{-1}$ ($1.52 \text{ kcal}\cdot\text{mL}^{-1}$) and 16% of energy as protein, 34% as fat, and 50% as carbohydrate. No other food and beverage (other than water) was consumed from this meal until the end of the cycling bout. Subjects ingested 0.5–1 L of water per hour of cycling.

Supplementation. Subjects ingested the supplements daily for 6 wk. Under double-blind procedures, subjects assigned to placebo and n-3fa ingested four soft gel capsules per day (two in the morning on an empty stomach at 7:00–8:00 a.m. and two at 6:00–8:00 p.m. before the evening meal) for 6 wk before and during a 3-d period of intensified exercise. The n-3fa capsules contained a high-potency fish oil concentrate from anchovy and sardines, with soybean oil, natural flavors, tocopherols, canola oil, and citric acid, and provided 2000 mg of EPA and 400 mg of DHA (in four soft gels). The placebo capsules were identical in appearance and contained the same ingredients but without the fish oil concentrate. The multivitamin–mineral portion of the supplement contained a mixture of vitamins and minerals with an emphasis on antioxidants including vitamins C, E, and A. Subjects assigned to vitamin and minerals ingested eight tablets per day (four in the morning on an empty stomach at 7:00–8:00 a.m. and four tablets at 6:00–8:00 p.m. before the evening meal) for 6 wk before and during a 3-d period of intensified exercise. A total of eight tablets provided vitamins C (2000 mg), E (800 IU), and A (3000 IU) and selenium (200 μg). Subjects on combined n-3fa and vitamin and minerals took a combination of two n-3fa softgels and four vitamin and mineral tablets twice daily as described. To ensure compliance, subjects were called weekly and e-mailed daily for reminders and required to return the empty supplement boxes.

Blood and exercise. Baseline blood samples (blood 1) were collected between 7:30 and 9:00 a.m. after an overnight fast and before supplement ingestion. Before exercise and after 6 wk of supplementation, subjects provided blood samples at 8:00 a.m. after an overnight fast and having avoided exercise training for at least 12 h and before having ingested the morning dose of supplements (blood 2). Subjects then came back to the laboratory for three consecutive

days and cycled from 3:00 to 6:00 p.m. at approximately 57% maximal watts while continuing the daily supplement. During the test sessions, experimental subjects cycled using their own bicycles on CompuTrainer™ Pro Model 8001 trainers with the exercise load set at approximately 57% maximal watts. Metabolic measurements were made every 30 min of cycling using the MedGraphics CPX metabolic system to verify workload. Blood samples were obtained within 15 min after completing the third 3-h exercise bout (6:00 p.m.) (blood 3).

Blood sampling conditions and disposition. Blood samples were drawn from an antecubital vein with subjects in the supine position. A local clinical hematology laboratory used a Coulter STKS instrument (Coulter Electronics, Hialeah, FL) and provided hemoglobin and hematocrit for determination of plasma volume change using the method of Dill and Costill (9). Other blood samples were centrifuged in sodium heparin or EDTA tubes, and plasma was aliquoted and then stored at -80°C before analysis for plasma EPA and DHA, F_2 -isoprostanes, and plasma antioxidant parameters (ferric-reducing antioxidant potential (FRAP) and oxygen radical absorption capacity (ORAC)).

F_2 -isoprostanes. Plasma F_2 -isoprostanes were determined using gas chromatography mass spectrometry (GC-MS) as previously described (21). Briefly, free F_2 -isoprostanes were extracted from plasma added to a deuterated [2H4] PGF2 internal standard. The mixture was then added to a C18 Sep Pak column, followed by silica solid phase extractions. F_2 -isoprostanes were converted to pentafluorobenzyl esters, subjected to thin layer chromatography, and converted to trimethylsilyl ether derivatives. Samples were analyzed by a negative ion chemical ionization GC-MS using an Agilent 6890N gas chromatography interfaced to an Agilent 5975B inert MSD mass spectrometer (Agilent Technologies, Inc. Santa Clara, CA). Select ion monitoring techniques were used to analyze the derivatives of F_2 -isoprostanes and the internal standard. The height of the two ions monitored (m/z 569 for F_2 -isoprostanes and m/z 573 for the internal standard, respectively) were compared to give a concentration of F_2 -isoprostane in plasma in nanograms or picograms per milliliter.

Plasma EPA and DHA. After addition of 500 μg of butylated hydroxytoluene and 20 μg of heptadecanoic acid methyl ester (NU-Chek Prep, Inc. Elysian, MN) internal standard to 100 μL thawed plasma, lipids were extracted,

and methyl esters were formed after the mixture was added to 2 mL of methanolic 5% HCl and incubated at 80°C for 2 h in an OLS200 Shaking Waterbath (Grant Instruments Ltd., Shepreth, Cambridgeshire, SG8 6 GB England). The samples were cooled to room temperature upon completion of the incubation, and the methyl esters were extracted twice with 2 mL of n-hexane. The top layer of the supernatant was combined and dried with N-EVAP116 Nitrogen Evaporator (Organomation Associates, Inc. Berlin, MA). The dried extract was then reconstituted in 200 μL of n-hexane supplemented with 0.05% butylated hydroxytoluene, of which 1 μL was injected into an HP 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a 5975B Inert XL MSD mass spectrometer detector. A DB-WAX GC column (30 m \times 320 μm \times 0.25 μm) from J & W Scientific (Agilent Technologies) was used to separate the methyl esters of the extracted fatty acids. Fatty acid concentrations were calculated in relation to the heptadecanoic acid methyl ester internal standard peak. The concentration of each phospholipid fatty acid was expressed as a concentration ($\mu\text{g}\cdot\text{mL}^{-1}$ plasma).

Ferric-reducing antioxidant potential. Total plasma antioxidant potential was determined by the ferric-reducing antioxidant potential (FRAP) assay according to the methodology of Benzie and Strain (3). The basis of this assay is that water-soluble reducing agents (antioxidants) in the plasma will reduce ferric ions to ferrous ions, which then react with an added chromogen. Samples and standards were analyzed in duplicate and expressed as ascorbate equivalents on the basis of an ascorbate standard curve (0–1000 μmol). Intraassay and interassay coefficients of variation were less than 5% and 7%, respectively.

Oxygen radical absorption capacity (ORAC). The ORAC assay was a modification of the methodology of Ou et al. (24). Briefly, trolox standards were made from a trolox stock solution. A serial dilution of a 50- μM trolox solution was made with a phosphate-buffered solution to produce 25, 12.5, and 6.25 μM of trolox standards. A fluorescein (Sigma-Aldrich Chemicals, St. Louis, MO) working solution was made by pipetting 800 μL of stock solution into 50 mL of phosphate buffer in a 50-mL conical tube. Before use, the solution was incubated in the water bath at 37°C until thoroughly heated.

The AAPH solution was made by dissolving 0.108 g of AAPH (Wako Chemical, Richmond, VA) into 5 mL of

TABLE 1. Subject characteristics for baseline measures (values are presented as mean \pm SEM).

Variable	P (n = 12)	VM (n = 12)	N3 (n = 11)	VN3 (n = 13)	P Value
Age (yr)	26.9 \pm 2.8	22.2 \pm 0.9	24.1 \pm 2.4	25.2 \pm 2.1	0.460
Body mass (kg)	67.4 \pm 3.8	73 \pm 3.5	68.9 \pm 3.0	66.7 \pm 4.0	0.761
$\dot{V}\text{O}_{2\text{peak}}$ ($\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$)	58.8 \pm 3.1	66.6 \pm 3.1	62.9 \pm 3.8	59.8 \pm 7.3	0.408
Power _{max} (W)	251 \pm 18.5	309 \pm 14.6	284 \pm 13.9	281 \pm 16.8	0.172
HR _{max} ($\text{beats}\cdot\text{min}^{-1}$)	185 \pm 2.8	187 \pm 2.2	188 \pm 3.0	187 \pm 3.0	0.362
Body composition (% fat)	16.4 \pm 2.7	14.5 \pm 1.8	13.7 \pm 1.8	14.8 \pm 1.7	0.431
BMI ($\text{kg}\cdot\text{m}^{-2}$)	21.1 \pm 1.2	23.1 \pm 1.2	21.7 \pm 0.7	21.7 \pm 1.2	0.671
Training ($\text{km}\cdot\text{wk}^{-1}$)	209 \pm 36.2	240 \pm 31.4	213 \pm 38.5	264 \pm 38.5	0.681
Training ($\text{h}\cdot\text{d}^{-1}$)	0.64 \pm 0.13	1.14 \pm 0.22	1.09 \pm 0.17	1.02 \pm 0.19	0.173

P, placebo; VM, vitamin–mineral; N3, n-3 fatty acid; VN3, vitamin–mineral and n-3 fatty acid combination.

TABLE 2. Performance measures for average of three exercise sessions (values are presented as mean \pm SEM).

Variable	P (n = 12)	VM (n = 12)	N3 (n = 11)	VN3 (n = 13)	P Value
Watts	143 \pm 10.3	175 \pm 8.5	162 \pm 8.1	160 \pm 9.9	0.102
Power (% W_{max})	56.8 \pm 0.2	56.7 \pm 0.3	56.8 \pm 0.2	57.1 \pm 0.2	0.822
Mean HR (beats·min ⁻¹)	144 \pm 3.4	137 \pm 1.8	141 \pm 2.2	139 \pm 2.6	0.322
HR (% HR _{max})	78.2 \pm 1.1	73.4 \pm 1.0	74.8 \pm 1.4	74.6 \pm 1.9	0.069
Mean $\dot{V}O_2$ (mL·min ⁻¹)	2589 \pm 180	3025 \pm 153	2725 \pm 170	2846 \pm 193	0.590
$\dot{V}O_2$ (% $\dot{V}O_{2peak}$)	66.3 \pm 3.0	63.2 \pm 2.2	64.2 \pm 3.2	65.1 \pm 3.7	0.642
RPE (rating perceived exertion)	13.0 \pm 0.3	12.9 \pm 0.3	13.4 \pm 0.4	12.9 \pm 0.4	0.136

P, placebo; VM, vitamin–mineral; N3, n-3 fatty acid; VN3, vitamin–mineral and n-3 fatty acid combination.

incubated phosphate buffer immediately before the start of the assay. The microplate was prepared and loaded in a “forward-then-reverse” order to avoid possible positional errors. The edge wells were left empty or blank (phosphate-buffered working solution) to reduce the impact of “edge effect” on samples and standards particularly from temperature effects on the outside wells. Twenty microliters of sample, blank, and trolox standard solutions were pipetted into appropriate wells. Then, 200 μ L of fluorescein working solution was added to each well using an eight-channel micropipettor. A cover was placed on the microplate, and the plate and the contents were incubated at 37°C for at least 20 min. Then, 20 μ L of AAPH working solution was added using an eight-channel micropipettor as quickly as possible. Final ORAC values (μ m·L⁻¹ trolox) were made from the plate reader-derived area under the curve. Then, the final ORAC_{FL} values were calculated by using a quadratic regression equation $x = -b \pm \sqrt{b^2 - 4ac} + 4cy$. Excitation wavelength was 485 nm, and emission wavelength was 520 nm.

Statistical analysis. Data are expressed as mean \pm SEM. All data were screened for outliers with outliers designated as being outside of the mean \pm 2.5 SD. Data in Tables 1 and 2 were compared between groups using Student’s *t*-tests. All other data (Figs. 1–5) were analyzed using a 4 (groups) \times 3 (time points) repeated-measures ANOVA to determine overall main effects. To account for multiple comparisons

between treatments, a Bonferroni adjustment was made and the significant *P* value established as $P \leq 0.017$ and the data compared using one-way ANOVA. These data analysis allowed group comparisons for two different effects: 6 wk of chronic supplementation and acute postexercise changes.

RESULTS

Descriptives. Subject baseline characteristics are summarized in Table 1. Outcome data for male and female cyclists did not differ significantly and are presented together for each group. During the 3-h cycling bouts, temperature averaged 22.4°C \pm 0.06°C and relative humidity 35.8% \pm 1.6%. No significant differences were found among groups for basic physical characteristics (Table 1). Subject performance characteristics were averaged over the three exercise sessions and are presented in Table 2. There was no effect of treatment group on any exercise related performance variable. Plasma volume change did not differ between groups postexercise on the third day and averaged less than 2% because of ingestion of 0.5–1.0 L of water per hour of exercise (data not shown).

Oxidative, n-3fa, and antioxidant variables. EPA and DHA supplementation resulted in drastic increases from baseline to preexercise. DHA main effects were treatment ($P = 0.068$), time ($P = 0.004$), and interaction ($P = 0.006$).

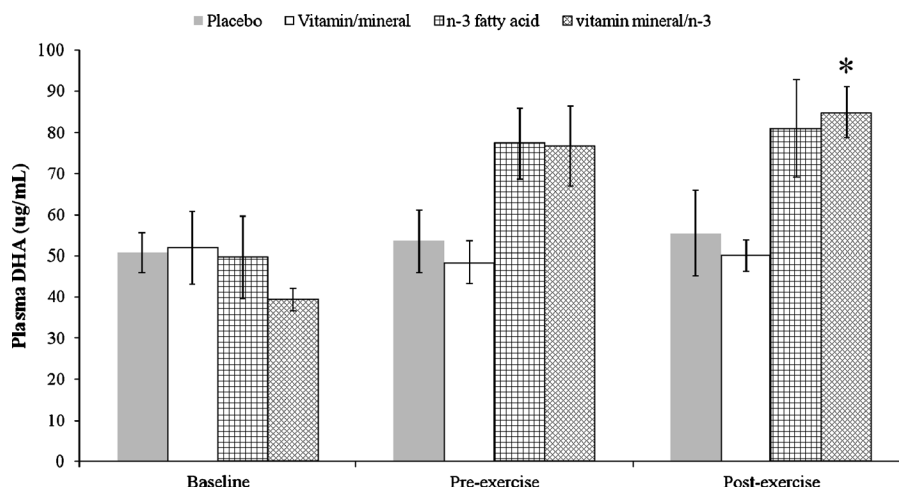


FIGURE 1—Plasma DHA values over time among four treatments. Treatment effect ($P = 0.184$), time effect ($P = 0.002$), and treatment \times time interaction ($P = 0.006$). * $P \leq 0.0125$ after Bonferroni correction versus placebo value at baseline ($n = 8$). Values are presented as mean \pm SEM.

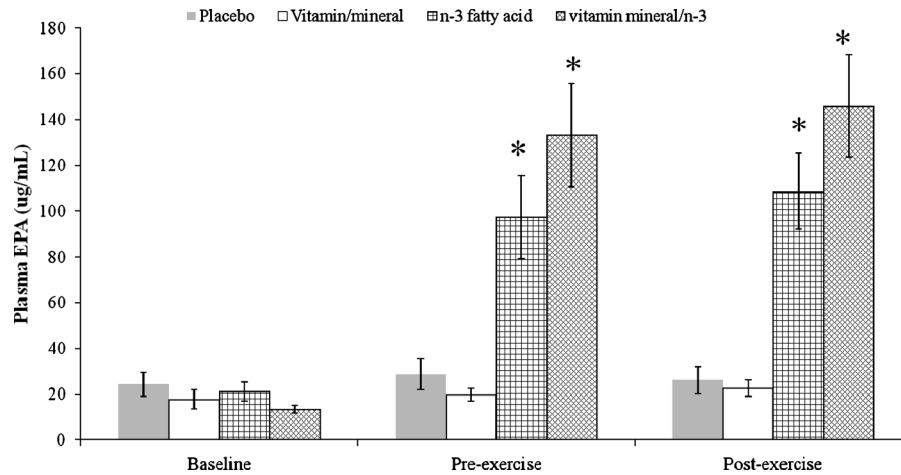


FIGURE 2—Plasma EPA values over time among four treatments. Treatment effect ($P = 0.001$), time effect ($P = 0.001$), and treatment \times time interaction ($P = 0.001$). * $P \leq 0.0125$ after Bonferroni correction versus placebo value at baseline ($n = 9$). Values are presented as mean \pm SEM.

Plasma DHA increased 55.8% in N3 and 95.3% in VN3 from baseline to preexercise, respectively (Fig. 1). EPA exhibited significant treatment ($P \leq 0.001$), time ($P \leq 0.001$), and interaction effects ($P \leq 0.001$). Plasma EPA increased 61.5% in N3 and 778% in VN3 from baseline to preexercise (Fig. 2). The change in F_2 -isoprostanes produced significant overall treatment effects ($P = 0.014$) and time effects ($P = 0.001$). Interestingly, F_2 -isoprostanes increased approximately 53% from baseline to immediately postexercise in N3. This change was significantly higher versus the change observed in P ($P \leq 0.01$). VM and VN3 groups were not different from P (Fig. 3). When specific comparisons of the pattern of change in F_2 -isoprostanes from baseline to postexercise were made between treatments, F_2 -isoprostanes in N3 were higher versus P ($P = 0.020$), higher versus VM ($P = 0.008$), and higher versus VN3 ($P = 0.05$) (Fig. 3).

ORAC declined similarly and significantly among all groups ($P \leq 0.001$) from preexercise, but the pattern of change was not different between groups ($P = 0.217$). The

exercise-induced decline was greatest in P (37%) versus N3 (23%), VM (23%), and VN 3 (21%) (Fig. 4). FRAP plasma antioxidant capacity values were higher after exercise among all groups. However, only the vitamin and mineral containing groups exhibited significant differences from baseline ($P \leq 0.017$) after Bonferroni correction. Overall effects for FRAP values were treatment ($P = 0.300$), time ($P = 0.003$), and interaction ($P = 0.045$). FRAP increased 26% immediately postexercise in VN 3, 19% in VM, 5.9% in N3, and 9.5% in P (Fig. 5).

DISCUSSION

One of the most important outcomes of this investigation was that consumption of a supplement containing 2000 mg of EPA and 400 mg of DHA daily for 6 wk and during 3 d of intense exercise resulted in a significantly larger increase (53% increase from preexercise) in F_2 -isoprostanes compared

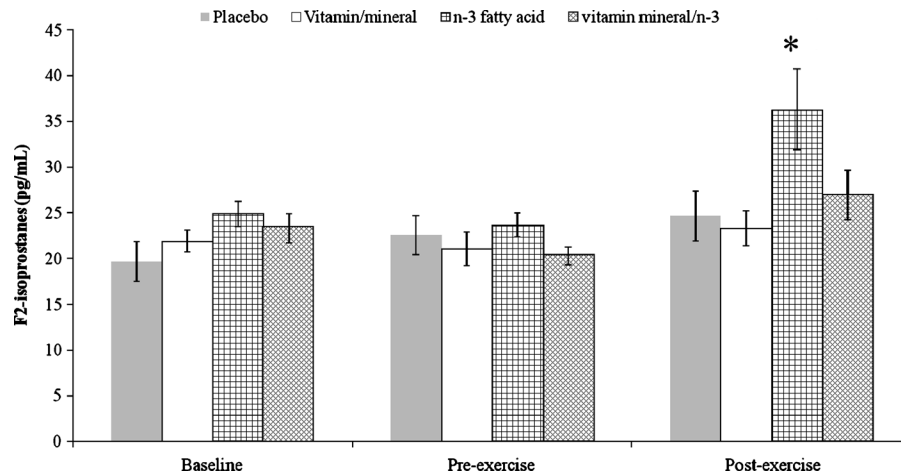


FIGURE 3— F_2 -isoprostane values over time among four treatments. Treatment effect ($P = 0.014$), time effect ($P = 0.002$), and treatment \times time interaction ($P = 0.224$). * $P \leq 0.0125$ after Bonferroni correction versus placebo value at baseline ($n = 12$). Values are presented as mean \pm SEM.

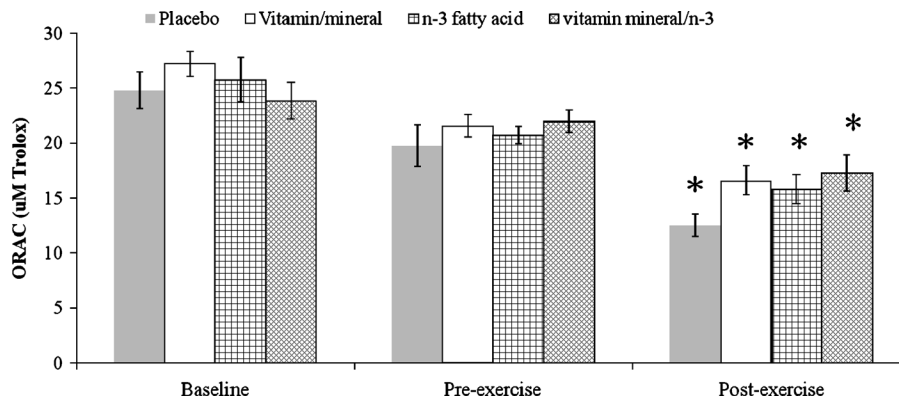


FIGURE 4—Plasma ORAC values over time among four treatments. Treatment effect ($P = 0.187$), time effect ($P = 0.001$), and treatment \times time interaction ($P = 0.381$). * $P \leq 0.0125$ after Bonferroni correction versus placebo value at baseline ($n = 10$). Values are presented as mean \pm SEM.

with placebo and other antioxidant and n-3fa combinations after exhaustive exercise. This is of some importance given that F_2 -isoprostanes are bioactive and elevated in obesity and various other disease states (20). This outcome supports previous concerns that an increased ingestion of polyunsaturated fatty acids, whether n-3fa or n-6fa, might lead to increased lipid peroxidation (19). As further evidence that n-3fa enhanced lipid peroxidation in our study, F_2 -isoprostanes were only increased by 10% or less within the treatment groups that did not contain n-3fa. These smaller changes in F_2 -isoprostanes are similar to other studies we have conducted, indicating that longer duration exercise (exceeding 1 h) does not increase F_2 -isoprostanes to the degree of shorter intense exercise (17,23).

Several interesting points emerge from our investigation. Although it is clear that increased n-3fa ingestion can result in increased lipid peroxidation (11,29), the n-3fa peroxidation products do not directly result in production of F_2 -isoprostanes. Instead, the main products are F_3 -isoprostanes (from EPA) and F_4 -isoprostanes (from DHA) (30) as well as the aldehyde fragment 4-hydroxy-2E-hexenal (11). These compounds have been found to be increased in tissues submitted to oxidative stress (11,30). As F_2 -isoprostanes derive solely from the oxidation of arachidonate (20), this implies

that downstream n-3fa peroxidation products or some other mechanism increases F_2 -isoprostanes indirectly. In support of this hypothesis, Catala (6) states that lipid hydroperoxides can participate in chain reactions that increase damage to other lipid biomolecules. Most hydroperoxides are reduced to corresponding alcohols by glutathione peroxidases but some degrade into other compounds, including hydroxy-alkenals. The best known of these are 4-hydroxy-2-nonenal (4-HNE) and 4-hydroxy-2E-hexenal, which derive from lipid peroxidation of n-6fa and n-3fa, respectively. These aldehydes are relatively stable and can diffuse within or escape from the cell and attack other biomolecules distant from the original event. These aldehydes exhibit great reactivity with proteins, DNA, and membrane bound phospholipids. Arachidonic acid is prominent in membrane phospholipids that would oxidize directly to produce F_2 -isoprostanes (20).

Another observation is that n-3fa supplementation typically reduces baseline concentrations of F_2 -isoprostanes (19). Nalsen et al. (22) found that supplementation with a total of 3.6 g n-3fa (2.4g EPA and DHA combined) per day significantly reduced F_2 -isoprostane concentrations in non-exercising healthy humans after 3 months versus placebo. Mori et al. (19) provided daily fish meals or EPA and DHA

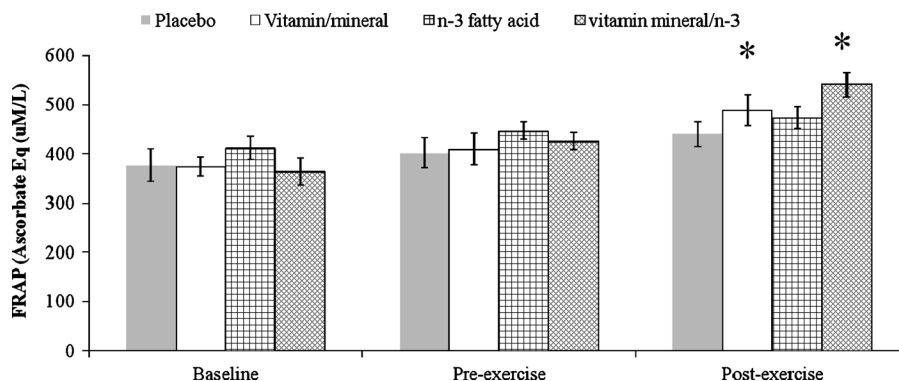


FIGURE 5—FRAP values over time among four treatments. Treatment effect ($P = 0.409$), time effect ($P = 0.002$), and treatment \times time interaction ($P = 0.035$). * $P \leq 0.0125$ after Bonferroni correction versus placebo value at baseline ($n = 12$). Values are presented as mean \pm SEM.

and found that urinary F₂-isoprostanes were significantly reduced by 20%–27% in both trials. We did not observe this decrease from baseline with 6 wk of supplementation. An interesting difference is that our entire subject population were athletes engaged in routine heavy endurance exercise versus those of Mori et al. (19) and Nalsen et al. (22).

Because the concentration of F₂-isoprostanes was so drastically increased postexercise versus other treatments, we hypothesize that consumption of high amounts of n-3fa (14) does not increase peroxidation until a state of high oxidative stress is initiated. Excessive radical formation and trauma during exercise leads to an inflammatory state that may propagate overall lipid peroxidation and increase F₂-isoprostanes as addressed earlier. Because n-3fa are apparently susceptible to oxidation during exercise, it becomes important whether the chain reaction of lipid peroxidation can be interrupted by increased dietary antioxidants and whether increased concentrations of n-3fa in the cellular membrane increase antioxidant requirements. F₂-isoprostanes were increased 53% by n-3 fatty acids alone but only 32.8% by the same amount of n-3fa coupled with large-dose antioxidant vitamins. Interestingly, this suggests that coingestion of antioxidants with the n-3fa offered some protection from oxidation. Although using a less sensitive indicator of lipid peroxidation, Allard et al. (1) examined supplementation with a total of 6.26 g of EPA and DHA n-3fa daily for 6 wk in 80 healthy men alone or in combination with alpha-tocopherol (900 IU of vitamin E) versus placebo on lipid peroxidation. Supplementation with n-3fa significantly increased plasma MDA and lipid peroxides. However, consumption of vitamin E concurrent with the n-3fa did not reduce the increase in lipid peroxidation. In contrast, we used combinations of antioxidants added to the n-3fa supplement, which may have been more effective and accounted for the attenuation in F₂-isoprostanes observed in the n-3 and antioxidant combination group (16).

FRAP plasma antioxidant capacity values were higher after exercise among all groups. However, only the vitamin and mineral containing groups exhibited significant differences from baseline. The FRAP assay has been found to respond strongly to the presence of water-soluble antioxidants such as urate and vitamin C (3). Interestingly, vitamin and mineral supplementation did not significantly increase ORAC value compared with placebo. Plasma ORAC values declined significantly and similarly between treatments after exercise, suggesting depletion of an antioxidant or multiple antioxidants, which was not prevented by antioxidant supplementation. The uniqueness of the ORAC assay is that total antioxidant capacity of a sample is estimated by taking an oxidation reaction to completion. ORAC measures all of the nonprotein antioxidants, which include alpha-tocopherol, glutathione, vitamin C, beta-carotene, uric acid, and bilirubin. In this regard, ORAC constitutes more of a comprehensive antioxidant measurement encompassing multiple antioxidants.

Whether strenuous exercise greatly increases the need for additional antioxidant vitamin and minerals in the diet or through supplementation is not clear. Interestingly, the opposite may be true because Gomez-Cabrera et al. (10) and Ristow et al. (25) found that supplementation with antioxidant vitamins actually reduced beneficial adaptations from exercise and therefore might be contraindicated. Nonetheless, as previously mentioned, we observed higher FRAP values postexercise in the groups receiving vitamin and minerals, but this did not affect F₂-isoprostanes. However, Mastaloudis et al. (16) found that 6 wk of supplementation with 1000 mg vitamin C and 300 mg RRR-alpha-tocopheryl acetate prevented exercise-induced F₂-isoprostanes but had no effect on inflammatory markers after a 50-km ultramarathon. McNulty et al. (17) examined the effect of 2 months of vitamin E supplementation (800 IU·d of alpha-tocopherol) in highly trained athletes during exhaustive exercise on plasma antioxidant potential and F₂-isoprostanes. Conversely, this study found that prolonged large doses of alpha-tocopherol supplementation did increase plasma antioxidant potential postexercise, but lipid peroxidation was actually higher in the supplemented group versus placebo. Nieman et al. (23) evaluated the influence of vitamin C supplementation (1500 mg·d⁻¹) during the 7-d period before the race and on race day compared with placebo on F₂-isoprostanes in runners competing in an ultramarathon race. Despite higher plasma vitamin C concentrations, no significant effects were measured for lipid hydroperoxides or F₂-isoprostanes. Evidently, multiple factors such as timing, type and concentration of antioxidants, and mode of exercise influence the outcomes associated with antioxidant supplementation.

In conclusion, despite the beneficial effects typically associated with n-3fa ingestion, we observe for the first time evidence that supplementation with n-3fa is associated with significantly increased F₂-isoprostanes in response to heavy cycling exercise. Supplementation with antioxidant vitamin combinations and minerals did not significantly influence F₂-isoprostane formation versus placebo. Furthermore, n-3fa supplementation did not influence any measure of plasma antioxidant capacity, but a vitamin and mineral supplementation was effective at increasing FRAP values. On the basis of our results, further assessment is clearly needed to examine whether the possible benefits of n-3fa supplementation would outweigh the potential risks of increased lipid peroxidation in athletes who are engaged in heavy exercise. Furthermore, because this study was conducted in a young fit population, future studies should include aging or older adults, whom often are prescribed exercise and fish oils to promote cardiovascular health because aging compromises antioxidant defenses (13).

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